RESEARCH PLAN PROPOSAL

STUDY OF THE EFFECT OF TITANIUM DIOXIDE NANOPARTICLES USED IN COSMETICS IN MICE AND PREVENTIVE ACTION OF ALOE VERA GEL

For registration to the degree of
Doctor of Philosophy
FACULTY OF LIFE SCIENCES

DEPARTMENT OF ZOOLOGY
THE IIS UNIVERSITY, JAIPUR

Submitted by
KRISHNA NAGER
ICG/2014/18533

SUPERVISOR:
Dr. PRIYANKA MATHUR
(Associate Professor)

CO-SUPERVISOR:
Dr. LATA SHAHANI
(Sr.Assistant Professor)

October, 2015-16

INTRODUCTION

There is a rapid development in the field of nanotechnology because of the quantum size and large surface area to volume ratio of nanomaterials, which gives them a unique property that can be compared with their larger counterparts. Nanotoxicological studies are intended to determine whether and to what extent these properties may pose a threat to the environment and to human beings. Nanotoxicology is a branch of bionanoscience which deals with the study and application of the toxicity of nanomaterials. (Buzea *et al.,* 2007) Nanomaterials even when made of immobile (inert) elements such as gold become highly active at nanometer dimensions thus play a remarkable role in toxicity, which is important for toxicologists, especially in respiratory diseases. (Jafar *et al.,* 2011) Some

studies on the different sizes of carbon and titanium oxide showed that reduction in nanoparticle size increases its toxicity in the lungs. Also notable is that combining some metals with each other causes complex toxicity which does not occur with single metals. (Jafar *et al.,* 2011)

In 1998 Zhang presented his findings on the effects of nanoparticles on respiratory toxicity and inflammation, some of the particle features such as size, surface chemistry, and oxidative stress functions play important roles in nanotoxicity. Other features such as crystalline, coating, and the longevity of particles have also been studied as important parameters. By gaining control over dangerous particles we can increase the use of nanoparticles by reducing their harmful effects, and thus allowing them to be used in the curing (remedial) of diseases.

The Royal Society identifies the potential for nanoparticles to penetrate the skin, and recommends that the use of nanoparticles in cosmetics be conditional upon favorable assessment by the relevant European Commission safety advisory committee. (Nel et al., 2006)

Nanoparticles are increasingly used in a wide range of applications in science, technology and medicine. (Cai *et al.,* 2008) Nanoparticles are particles between 1and100 nanometers in size. In nanotechnology a particle is defined as a small object that behaves as a whole unit with respect to its transport and properties. Nanoparticles are of interest because of the new properties such as chemical reactivity and optical behavior that they exhibit compared with larger particles of the same materials. For example titanium dioxide and zinc oxide become transparent at the nanoscale and have found application in sunscreens.

Nanoparticles have a range of potential applications viz. in cosmetics, textiles and paints as short term application and in the long term applications such as drug delivery where they could be used to deliver drugs to a specific site in the body. Nanoparticles can also be arranged into layers on surfaces, providing a large surface area and hence enhanced activity relevant to a range of potential applications such as catalysts. (Ambalavanan *et al.,* 2013)

Particles are further classified according to diameter. Ultrafine particles are the same as nanoparticles and between 1 and 100 nanometers in size. Coarse particles cover a range between 2,500 and 10,000 nanometers. Fine particles are sized between 100 and 2,500 nanometers. Nanoparticles have unique thermal, mechanical, magnetic and optical properties that allow for their widespread application in biomedicine and many industrial sectors. More than 1310 marketed consumer products are based on nanomaterials and this number is rapidly increasing. Nanoparticles cause pulmonary injury, hepatotoxicity, immunotoxicity, neurotoxicity, renal toxicity and reversible testis damage in animals. (Sun *et a.,l* 2013)

In cosmetic and skin care products titanium dioxide is used as a pigment, sunscreen and a thickener. It is also used as a tattoo pigment and in styptic pencils. Titanium dioxide is produced in varying particle sizes, oil and water dispersible, and in certain grades for the cosmetic industry.

Titanium dioxide is found in the majority of physical sunscreens because of its high refractive index, its strong UV light absorbing capabilities and its resistance to discolouration under ultraviolet light. This advantage enhances its stability and ability to protect the skin from ultraviolet light. Nano-scaled titanium dioxide particles are primarily used in sun screen lotion because they scatter visible light less than titanium dioxide pigments while still providing UVprotection. Sunscreens designed for infants or people with sensitive skin are often based on titanium dioxide or zinc oxide, as these mineral UV blockers are believed to cause less skin irritation than other UV absorbing chemicals. (Winkler, 2003)

REVIEW OF LITERATURE

The extensive application of nanomaterials in industry, medicine and consumer products has raised concerns about their potential toxicity. The female population is particularly vulnerable and deserves special attention because toxicity in this group may impact both female reproductivity and fetal development. (Sun *et al.,* 2013, Jafar *et al.,* 2011) Such adverse effects can be due to nanoparticle composition, surface modification, dose, exposure route and animal species. Mouse and zebra fish models due to their unique features are used to examine the potential toxicity of silver, titanium dioxide and zinc oxide nanoparticles.

Nanotechnology has wide applications in many fields, especially in the biological sciences and medicine. Nanomaterials are applied as coating materials or in treatment and diagnosis. Nanoparticles such as titanic, zirconia, silver, diamonds, iron oxides, carbon nanotubes, and biodegradable polymers have been studied in diagnosis and treatment but many of these nanoparticles may have toxic effects on cells that can be caused due to size, inherent properties, and surface chemistry. The toxicity can be reduced by biocompatible coated forms of nanoparticles.

Klaine *et al.,* 2008 in their study introduced the key aspects of nanoparticles in the environment-freshwater, marine and soil and discussed their fate, behavior, disposition and toxicity and those used in manufacture of products.

Since nanoparticles are produced for specific purposes which cannot be met by larger particles and that they are highly reactive, in particular, with biological systems. Kreyling *et al.,* 2006 study reveals that inhalation of nanoparticles leads to deposition in the various regions of the respiratory tract, systemic translocation and uptake in secondary target organs, epidemiologic associations with health effects and toxicology.

Studies of Kannan *et al.,* 2011 reveals that nanotoxicity was prevalent in the embryonic Zebrafish that showed many organogenesis deformities like cardiac malformations, eye and head edema, and tail and trunk flexure in the organ system of the developing embryos for 1 to 5 day post fertilization in different concentrations of Ag Nanoparticles. The Organogenesis disruptive effects were found in 14 - 20 ng/ml of silver nanoparticles but the inhibition was found in 4-10ng/ml for the pathogens in vitro and 10ng/ml in embryos. However in Cardiac assay, the Heart Beat rates were calculated as 42 - 45 for 15 Sec in the concentrations ranging from 10-20ng/ml of silver nanoparticles. The blood flows, rhythmicity, contractility of heart beat rates were observed normal. The Mean value of blood Cell counting did not show any notable effects in the Nanoparticle treated Zebrafish embryos and control. The LC50 value for the Biosynthesized nanoparticle was at22ng/ml in all the developmental stages of the embryos. Their results show that silver nanoparticles disrupt the normal organogenesis during development.

Nanoparticles are used in an increasing number of biomedical, industrial, and food applications, but their safety profiles in developing organisms, including the human fetus and infant, have not been evaluated. Titanium oxide (TiO_2) nanoparticles, which are commonly used in cosmetics, sunscreens, paints, and food, have been shown to induce emphysema and lung inflammation in adult mice. The study conducted by Ambalavanan *et al.,* 2012 hypothesized that exposure of newborn mice to TiO_2 induced lung inflammation and inhibited lung development. C57BL/6 mice were exposed to TiO_2 (anatase; 8–10 nm) nanoparticles by intranasal instillation as a single dose on postnatal day 4 or as three doses on postnatal days 4, 7, and 10 (each dose = 1 μg/g body wt).Measurements of lung function (compliance and resistance),development (morphometry), inflammation (histology; multiplex analysis of bronchoalveolar lavage fluid for cytokines; PCR array and multiplex analysis of lung homogenates for cytokines) was performed on postnatal day 14.It was observed that a single dose of TiO_2 nanoparticles led to inflammatory cell influx, and multiple doses led to increased inflammation and inhibition of lung development without significant effects on lung function. Macrophages were noted to take up the TiO_2 nanoparticles, followed by polymorphonuclear infiltrate. Multiple cytokines and matrix metalloproteinase-9 were increased in lung homogenates, and Vascular Endothelial Growth Factor (VEGF) was reduced. These results suggested that exposure of the developing lung to nanoparticles may lead to ineffective clearance by macrophages and persistent inflammation with resulting effects on lung development and may possibly impact the risk of respiratory disorders in later life.

Iavicoli *et al.,* (2013) A *in vitro* and *in vivo* studies have demonstrated that nanoparticles pose hazard to human health, particularly on the endocrine system. The aim was to understand the potential endocrine disrupting risks posed by nanoparticles, to assess their underlying mechanisms of action and identify areas in which further investigation is needed in order to obtain a deeper understanding of the role of nanoparticles as endocrine disruptors. Current data support the notion that different types of nanoparticles are capable of altering the normal and physiological activity of the endocrine system.

Nanotechnologies have led to their implementation for *in vivo* diagnostic and therapy. Thus, the main applications of inorganic nanoparticles are imaging for diagnosis and cell tracking, photothermal and drug-delivery therapies. Following nanoparticles *in vivo* administration, the systemic circulation can distribute them to every body organ and tissue. Precise characterization of nanoparticles distribution and accumulation in the different body parts in preclinical models is required before any application in humans Varna *et al.,* (2012).

Ernest *et al* (2011) Silver nanoparticles (AgNPs) are extensively used in most of the consumer products. The genotoxic effect by lymphocyte proliferation assay and hemolysis assay was studied metal ion analysis was also done to check the uptake of nanoparticles in the cell pellet of human peripheral blood cells to ensure the generation of radical oxygen species (ROS), a possible mechanism by which the DNA damage could happen. The results indicate that the aberrant cell damage for the exposure period of 24hrs was found to be 0, 8 and 24% for 5, 15 and 25μg/mL of Ag NPs respectively.

Interestingly, cytotoxicity studies on erythrocytes also reveal the toxic effect of silver nanoparticles with membrane damage of the lipo-polysaccharide layer through haemolysis assay. This research validates that even at 15 μg/mL concentrations of AgNPs (which releases 1.8 μg/mL of Ag ions) has the potential to cause toxicity as analyzed by a range of cytotoxic and genotoxicity parameters. The chromosomal aberrations and cell cycle arrest issues the safety related to AgNPs. Chromosomal aberrations are believed to be the key factors resulting in cell cycle arrest. AgNPs are proved to be cytotoxic and genotoxic. Prospective application of AgNPs as an anti-proliferative agent could be narrowed by the fact that it is similarly toxic to normal cells.

The chemical stability and nonallergic, nonirritant, and ultraviolet protective properties, titanium dioxide (TiO_2) nanoparticles (NPs) have been widely used in industries such as electronics, optics, and material sciences, as well as architecture, medicine, and pharmacology. However, increasing concerns have been raised in regards to its ecotoxicity and toxicity on the aquatic environment as well as to humans Liu *et al.,* (2013) Although insights have been gained into the effects of

TiO_2 NPs on susceptible biological systems, there is still much area to be covered, particularly on the effects of the interaction of TiO_2 NPs with other chemicals or physical factors. Studies suggest that interactions of TiO_2 NPs with other chemicals or physical factors may result in an increase in toxicity or adverse effects.

Zakhidov *et al.,* (2012) studied the response of the mouse male germ cells exposed to gold nanoparticles (~2.5 nm). The investigation demonstrated that treatment with Au nanoparticles for four days does not impair the architecture of the spermatogenic epithelium. Cytogenetic evaluation using micronucleus assay showed that gold nanoparticles can affect the chromosomes of early primary spermatocytes. However, gold nanoparticles did not induce chromosome abnormalities in spermatogonial stem cells. Further, the cauda epididymal sperm was isolated on the 14th day after treatment and was incubated in SDS solution (sodium dodecyl sulphate) and then in a solution containing DTT (dithiothreitol) to induce nuclear chromatin decondensation. Observations showed that after four days of treatment of spermiogenic (postmeiotic) cells with gold nanoparticles the decondensation process had no differences from the control. On the contrary, in the experiment with the same cells and period of fixation but with a single exposure to gold nanoparticles, the number of mature gametes with totally decondensed nuclei reached 100% as opposed to 44% in the controls.

Trouiller *et al.,*(2009) Titanium dioxide (TiO_2) nanoparticles are manufactured worldwide in large quantities for use in a wide range of applications including pigment and cosmetic manufacturing. Although TiO_2 is chemically inert, TiO_2 nanoparticles can cause negative health effects, such as respiratory tract cancer in rats. However, the mechanisms involved in TiO_2 induced genotoxicity and carcinogenicity has not been clearly defined and are poorly studied *in vivo*. The study investigated TiO_2 nanoparticles induced genotoxicity oxidative DNA damage, and inflammation in a mice model. Wild-type mice were treated with TiO_2 nanoparticles in drinking water and determined the extent of DNA damage using the comet assay, the micronuclei assay, and the γ-H2AX immunostaining assay and by measuring 8-hydroxy-2′-deoxyguanosine levels and, as a genetic instability endpoint, DNA deletions. mRNA levels of inflammatory cytokines in the peripheral blood were also determined. These results showed that TiO_2 nanoparticles induced 8-hydroxy-2′-deoxyguanosine, γ-H2AX foci, micronuclei, and DNA deletions. The formation of γ-H2AX foci, indicative of DNA double-strand breaks, was the most sensitive parameter. Inflammation was also present as characterized by a moderate inflammatory response.

Iavicoli *et al*., (2012) stated essence of nanotechnology is the production of nanoparticles with unique physicochemical properties allowing worldwide application in new structures, materials, and devices. The consequently increasing human exposure to NPs has raised concerns regarding their health and safety

profiles. Titanium dioxide (TiO_2) has been reported to induce adverse pulmonary responses in exposed animals. However the potential more dangerous biological activities of TiO_2 NPs compared to their fine-sized counterparts are not fully understood. Therefore, the work was aimed to provide a comprehensive evaluation of the toxic effects induced by TiO_2 NPs in *in vivo* experiments.

The interactions between nanoparticles (NPs) and proteins in complex biological application media such as blood serum are capable of inducing aggregate formation which can lead to subsequent changes in biological activity. Here, we correlate surface charge, aggregation-tendency, and surface serum protein adsorption with cellular uptake and bio distribution in mice. Polystyrene-based NPs (80 - 170 nm) with different surface function alizations were synthesized and incubated with human serum. Interaction of NPs with serum proteins and aggregate formation were analyzed by mass spectrometry analysis and dynamic light-scattering. Influence of surface function alization on specific cellular uptake and organ distribution was characterized. Localization and organ targeting of intravenously applied NPs preferentially depended on their aggregation behavior in the presence of serum. Whereas strongly aggregating particles mainly located to liver, non-aggregating particles distributed to all organs. Determination of aggregate formation of NPs in the presence of serum and further analysis of the protein corona allows for preselection of NPs for *in vivo* application (Mohr *et al.,* (2014).

TiO_2 has a wide range of applications in many fields. It is often considered to be physiologically inert to humans. However some recent studies have reported that nano sized TiO_2 may generate potential harm to the environment and humans. In this paper the *in vivo* acute toxicity of nano-sized TiO_2 particles to adult mice was investigated. Mice were injected with different dosages of nano sized TiO2 (0, 324, 648, 972, 1296, 1944 or 2592 mg kg–1).The effects of particles on serum biochemical levels were evaluated at various time points (24 h, 48 h, 7 days and 14 days). Tissues (spleen, heart, lung, kidney and liver) were collected for titanium content analysis and histopathological examination. Treated mice showed signs of acute toxicity such as passive behavior, loss of appetite, tremor and ethargy. Slightly elevated levels of the enzymes alanine amino transferase and aspartate amino transferase were found from the biochemical tests of serum whereas blood urea nitrogen was not significantly affected ($P < 0.05$).$TiO_2$was also deposited in liver, kidney and lung. Histopathological examinations showed that some TiO_2 particles had entered the spleen and caused the lesion of spleen. Thrombosis was found in the pulmonary vascular system, which could be induced by the blocking of blood vessels with TiO_2 particles. Moreover, hepatocellular necrosis and apoptosis, hepatic fibrosis, renal glomerulus swelling and interstitial pneumonia

associated with alveolar septal thickening were also observed in high-dose groups. Chen *et al.,*(2009).

Protective role of 2-imino-4-methyl-1, 2-dihydropyrimido [5, 4C] quinoline-5(6H)-one (IMDHPQ) in indium titanium oxide nanoparticles (In TiO_2 NPs) induced hepatotoxicity was analyzed.In TiO_2 NPs were synthesized and given orally to albino rats to assess their hepatotoxicity. NPs mediated oxidative stress and liver tissue pathology was analyzed. Altered antioxidants (GSH, GPx, and catalase) and, biochemical (SGOT, SGPT, ALP, total protein, and total bilirubin) and histopathological changes were observed due to the oxidative stress caused by In TiO_2 NPs. Varying effects of IMDHPQ on each parameter were observed in the present study. The altered parameters of in TiO_2 NPs exposed rats might be due to the oxidative stress caused by NPs and hepatoprotective or ameliorative efficacy of quinoline compound IMDHPQ on signaling and molecular mechanism needs further study Bheeman *et al.,* **(**2014)

Several *in vivo* studies suggest that nanoparticles have the ability to reach the brain tissue Fujioka *et al.,* (2014). Moreover, some nanoparticles can penetrate into the brains of murine fetuses through the placenta by intravenous administration to pregnant mice. But it is not clear whether the penetrated nanoparticles affect neurogenesis or brain function. Its effects on neural stem cells, we assayed a human neural stem cell (hNSCs) line exposed in vitro to three types of silica particles (30 nm, 70 nm, and <44 μm) and two types of titanium oxide particles (80 nm and < 44 μm). Results show that hNSCs aggregated and exhibited abnormal morphology when exposed to the particles at concentrations ≥ 0.1 mg/mL for 7 days. Furthermore all the particles affected the gene expression of Nestin (stem cell marker) and neurofilament heavy polypeptide (NF-H,neuron marker) at 0.1 mg/mL. In contrast, only 30-nm silica particles at 1.0 mg/mL significantly reduced mitochondrial activity. Particularly 30-nm silica particles exhibited acute membrane permeability at concentrations ≥62.5 μg/mL in 24 h. Although these concentrations are higher than the expected concentrations of nanoparticles in the brain from *in vivo* experiments in a short period, these thresholds may indicate the potential toxicity of accumulated particles for long-term usage or continuous exposure.

Gold nanoparticles have potential applications in biomedicine, but are unsafe to use. Zhang *et al.,* (2010) found that the toxic effect in *in vitro* studies may not reflect in vivo responses. Here, an animal toxicity study of 13.5 nm gold nanoparticles in mice was presented in which animal survival, weight, hematology, morphology, and organ index were characterized at different concentrations (137.5–2200 μg/kg) over 14–28 days. The results showed that low concentrations of gold nanoparticles do not cause an obvious decrease in body weight or appreciable toxicity, even after their breakdown *in vivo.* High concentrations of

gold nanoparticles induced decreases in body weight, red blood cells, and hematocrit. It was also found that gold nanoparticles administered orally caused significant decreases in body weight, spleen index, and red blood cells. Of the three administration routes, the oral and intraperitoneal routes showed the highest toxicity, and the tail vein injection showed the lowest toxicity. Combining the results of all of these studies, it was suggested that targeted gold nanopartices by tail vein injection may be suitable for enhancement of radiotherapy, photothermal therapy, and related medical diagnostic procedures.

OBJECTIVE

- To study the possible toxic effect of an important nanoparticle titanium dioxide used in cosmetics through blood and urine examination of the test model.
- To study the reversal of the effect caused by appling titanium dioxide by using *Aloe vera* gel
- To find its effects at different dose levels
- To find the role of *Aloe vera* in nullifying its effects

MATERIAL AND METHODS

Titanium Dioxide Nanoparticle as a Test substance:-

Titanium dioxide absorbs ultraviolet light; this property makes titanium dioxide useful in sunscreens.

These nanoparticles are photocatalysts, which means that they have the capability to use energy in light to catalyze reactions with other molecules at reduced temperatures.

Titanium dioxide reflects all colors in the visible light spectrum therefore the light reflected from titanium dioxide is white. This characteristic makes it useful as a white pigment in paints and may make for white residue on the skin.

Researchers are also developing methods to use the photocatalytic properties of titanium dioxide nanoparticles to destroy cancer tumors. They are delivering titanium dioxide nanoparticles to cancer tumors using targeted drug delivery methods, and then shining light on the tumor. The titanium dioxide nanoparticles use the energy from the light to add an electron to oxygen molecules, which proceed to destroy cancer cells.

Some companies use titanium oxide nanoparticles as part of a film that uses the energy in light to start the chemical reaction that kills bacteria on surfaces.

Aloe vera gel used as a Protective agent:-

Common name: *Barbados aloe*

Family: Xanthorrhoeaceae

Aloe vera is a one of the most powerful and well- known medicinal plant**.** Today it has a strong global reputation due to its widespread usage in cosmetic, particularly to treat burns and sunburn, to aid wound healing, and to fight against cell aging. *Aloe vera* has shown very good results in skin diseases and it is often taken as health drink. It is also found effective in treating wrinkles, stretch marks and pigmentations. It also seems to be able to speed wound healing by improving blood circulation through the area and preventing cell death around a wound. (Kim *et al.,* 2010)

Swiss albino mice as an Experimental Model:-

Scientists and researchers rely on mice for several reasons. Rodents are small, easily housed and maintained, and adapt well to new surroundings. They also reproduce quickly and have a short lifespan of two to three years, so several generations of mice can be observed in a relatively short period of time.

Most of the mice used in medical trials are inbred so that other than sex differences, they are almost identical genetically. This helps make the results of medical trials more uniform, according to the National Human Genome Research Institute. As a minimum requirement mice used in experiments must be of the same purebred species.

Experimental Animal:- Swiss Albino Mice 6 weeks old will be procured from the animal house approved by the Ethical Committee CPCSEA Registration number No: 1689/PO/9/13/CPCSEA.

Drug:-Sub-Chronic Administration of Nanoparticle Titanium Dioxide (TiO2) in different concentrations.

Dose Administration:- Dose will be applied to a small area of skin (approximately 2 cm^2) of an experimental animal.

Dose:-

Dose1: - Sub-chronic exposure of 0.5 mg Titanium Dioxide.

Dose 2:- Sub-chronic exposure of 1.0 mg Titanium Dioxide.
Dose 3:- Sub-chronic exposure of 2.0 mg Titanium Dioxide.
Sample Collection: - 14 Days, 28 Days, 56 Days
Preventative Action of *Aloe vera Gel*
There will be five groups for experiments:-
a. Groups I: Normal Control (Untreated)
b. Groups II: Positive Control
3. Groups III: (Treated)
a. Experiment Group A1 (Dose: 0.5 mg of TiO_2)
b. Experiment Group A2 (Dose: 0.5 mg of TiO_2 +*Aloe vera* gel)
4. Groups IV:
a. Experiment Groups B1 (Dose: 1.0 mg of $TiO_{2)}$
b. Experiment Group B2 (Dose: 1.0 mg of TiO_2 +*Aloe vera* gel)
5. Groups V:
a. Experiment Groups C1 (Dose: 2.0 mg of TiO_2)
b. Experiment Group C2 (Dose: 2.0 mg of TiO_2 +*Aloe vera* gel)

Parameters:-

I. Blood Parameter: -

II. Skin Test:-

III. Urine Test:

IV. Histopathololology of Skin:

Significance of Parameters:-

I. Blood Parameter: -

1. Differential Leukocyte Count (DLC):-

A blood differential also called a white blood cell count differential measures the number of each of the five types of white blood cells present in our blood neutrophils, lymphocytes, monocytes, eosinophils, basophils. It can detect abnormal or immature cells and can be used to diagnose an infection, inflammation, leukemia, or an immune system disorder.

2. Hemoglobin:-

(Sahli's Haemoglobinometer or Haemometer Method) Hemoglobin is measured to detect anemia and its severity and to monitor anemic patient. The hemoglobin test measures the amount of hemoglobin in blood and is a good measure of the blood's ability to carry oxygen throughout the body.

3. Red Blood Cell (RBC):- (Neubar Chamber Method)

The method involves an accurate dilution of a measured quantity of blood with a fluid which is isotonic with the blood and which will prevent its coagulation. A dilution of 1 to 200 is usually necessary. The dilution blood is placed in a counting chamber and the number of cells is enumerated under a microscope.

The blood specimen is diluted (usually 200 times) with red cell diluting fluid which does not remove the white blood cells but allows the red cells to be counted under magnification in a known volume of fluid. Finally, the number of cells in undiluted blood is calculated and reported as the number of red cells/µl of whole blood.

4. White Blood Cell (WBC):- (Neubar Chamber Method)

A sample of whole blood is mixed with a weak acid solution that lyses no nucleated red blood cells. Following adequate mixing, the specimen is introduced into a counting chamber where the white blood cells (leukocytes) in a diluted volume are counted.

$$\text{WBCs per cu mm} = \frac{\text{Average number of chamber (2) WBCs count x dilution (20)}}{\text{Volume}}$$

White blood cells, also called leukocytes, are a major component of the body's immune system. White blood cells can help find an infection, an allergic or toxic reaction to medicines or chemicals, and many conditions, such as leukemia.

5. Total Blood cell (TBC/CBC) :- (Haemocytometer)

The total count of the red blood corpuscles and white blood corpuscles will be made with the aid of haemocytometer. A complete blood count (CBC) gives important information about the kinds and numbers of cells in the blood, especially red blood cells and white blood cells and platelets.CBC check any symptoms, such

as weakness, fatigue, or bruising etc.CBC also helps him or her diagnose conditions, such as anemia, infection, and many other disorders.

5. Mean corpuscular volume (MCV):-

Mean corpuscular volume (MCV) is a measure of the actual volume occupied by a red blood cell. This measurement is clinically significant in the presence of anemia as it allows for categorizing the type of anemia. The MCV shows the size of the red blood cells.

Formula:

6. Mean corpuscular hemoglobin concentration (MCHC):-

The MCHC measures the concentration of hemoglobin in an average red blood cell. These numbers help in the diagnosis of different types of anemia. Normal To calculate the MCHC, expressed as grams of hemoglobin per 100 ml packed cells, the following formula is used: -

Formula:

7. Haematocrit:-

This test measures the amount of space (volume) red blood cells take up in the blood. The value is given as a percentage of red blood cells in a volume of blood. For example, a hematocrit of 38 means that 38% of the blood's volume is made of red blood cells. Hematocrit and hemoglobin values are the two major tests that show if anemia or polycythemia is present.

$$\text{Haematocrit(Value \%)} = \frac{\text{Length of packed blood cell column after centrifugation (mm)}}{\text{Total length of blood column before centrifugation}} \times 100$$

Blood serum (SGOPT, SGPT, ALP, and ACP):-

A. Serum Glutamic Oxaloacetic Transaminase (SGOT)
B. Serum Glutamate Pyruvte Transaminase (SGPT)
C. Alkaline Phosphatase (ALP)
D. Acid Phosphatases (ACP)

A. Serum Glutamic Oxaloacetic Transaminase (SGOT):-
(International Federation of Clinical Chemistry (IFCC) Method for the determination of SGOT (ASAT) activity in serum)
The SGOT level is measured to check the function of liver, kidney, heart, pancreas, muscles, and red blood cells. It is also measured to monitor medical treatments that may lead to liver inflammation.

B. Serum Glutamate Pyruvte Transaminase (SGPT):-
(International Federation of Clinical Chemistry (IFCC) Method for the determination of SGPT (ASAT) activity in serum)
This test measures the amount of an enzyme called glutamate pyruvate transaminase (GPT) in the blood. This enzyme is found in many body tissues in small amounts, but it is very concentrated in the liver. It is released into the blood when cells that contain it are damaged. This enzyme is also called alanine transaminase, or ALT.
C. Alkaline Phosphatase (ALP):-
(Alkaline Phosphatase DGKC Kinetic Method)
The enzyme catalyses the hydrolysis of monophosphates at an alkaline pH. In the pastvarious substrates were used (including glycerophosphate, phenylphosphate), according to the recommendation by DGKC which is a kinetic method. The alkaline Phosphatase presents in the sample catalyses the hydrolysis of p-nitrophenylphosphate (pNPP) during which p-nitrophenol and phospahatase are released. Mg++ ions enhance activity. The increase in absorbance at 405 nm correlates with the activity of serum alkaline phosphatase.

D. Acid Phosphatase (ACP):- (Colorimetric Assay Kit)
Acid phosphatase assay kit is a high sensitivity, simple, direct and HTS-ready colorimetric assay designed to measure ACP activity in serum and other samples. It is suitable for research and drug discovery. The kit uses p-nitrophenyl phosphate (pNPP) as a phosphatase substrate which turns yellow (λmax = 405 nm) when dephosphorylated by ACP. The kit can detect μU acid phosphatase activity in samples.
II. Skin Test:-
1. Skin Absorbance (penetration) Test:- As per organisation for Economic Cooperation and Development (OECD) Guidlines).
Skin penetration testing also known as penetration, measures the absorption or penetration of a substance through the skin barrier and into the skin. Dermal penetration studies are conducted to determine how much of a chemical penetrates the skin, and thereby whether it has the potential to be absorbed into the systemic

circulation. Dermal absorption can occur from occupational, environmental, or consumer skin exposure to chemicals, cosmetics, and pharmaceutical products. Blood samples are taken and analyzed at different intervals of time.

2. Skin Irritation Test:- (Organisation for Economic Cooperation and Development (OECD) Guidelines).

Topical exposure to chemicals can lead to adverse skin effects. According to the severity and reversibility of effects one distinguishes skin corrosion (=skin burns) from skin irritation. Corrosive substances irreversibly damage the skin beyond repair, while irritant substances lead to a reversible local inflammatory reaction caused by the innate (non-specific) immune system of the affected tissue: in response to chemically induced tissue trauma and cell damage.

III. Urine Test:-

1. Creatinine Test: - (Creatinine Test Kit (JAFFE) Method)

At alkaline pH values, creatinine reacts with picric acid to produce a coloured compound, creatinine alkaline picrate, which is photometrically measured. Reagent kit for the determination of creatinine concentration in urine. Acolorimetric, alkaline picrate method (Jaffé).

2. Urea Test: - (Glutamate Dehydrogenase (GLDH) Kinetic Method)

Urea is acted upon by urease releasing ammonia and carbondioxide. The ammonia generated is utilized by glutamate Dehydrogenase (GLDH) in the presence of 2-Oxoglutarate (á-KG) to form glutamate. Simultaneously converting NADH to NAD resulting in a decrease in absorbance at 340nm. The rate of decrease in absorbance per minute is measured at 340nm.

IV. Histopathology of skin:

Histopathology (compound of three Greek words: *histos* "tissue", *pathos* "suffering", and *logia* "study of") refers to the microscopic examination of tissue in order to study the manifestations of disease. Specifically, in clinical medicine, histopathology refers to the examination of a biopsy or surgical specimen by a pathologist, after the specimen has processed and histological sections have placed onto glass slides.

PLAN OF WORK

1. Experiment Group I (0.5 mg Dose)
2. Experiment Group II (1.0 mg Dose)
3. Experiment Group III (2.0 mg Dose)

Sample collection (Blood and Urine) after

Important Features of the Experiment protocol:-

- Doses 0.5 mg, 1.0 mg, 2.0 mg of nanoparticle titanium dioxide (Tio_2) have been decided based on OECD Guidelines.
- Each day test dose will be applied three times a day at an interval of two hours.
- Protective gel of *aloe vera* of fixed concentration will be applied only once a day at fixed time to all the dose groups.
- Each day weight and photograph will be taken to record any effect on skin.

SIGNIFICANCE OF THE STUDY

The study will help in identifying the toxic nature of most prevalently used nano substance titanium dioxide in cosmetics and its implications and further in curing and nullifying these effects with the commonly used medicinal plant *Aloe vera.*

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